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Sulfide-Stimulation of Oxygen Consumption Rate and Cytochrome Reduction in Gills of the Estuarine Mussel *Geukensia demissa*

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Abstract. Organisms, such as the mussel *Geukensia demissa*, that inhabit high-sulfide sediments have mechanisms that impede sulfide poisoning of aerobic respiration. Oxygen consumption rates (nO_2) of excised ciliated gills from freshly collected *G. demissa* were stimulated 3-fold at sulfide concentrations between 200 and 500 μM and remained stimulated at 1000 μM. Maintenance of mussels in sulfide-free conditions resulted in less stimulation of gill nO_2 at <500 μM sulfide and inhibition between 500 and 1000 μM sulfide. Gills of *Mytilus galloprovincialis* from a sulfide-free environment were inhibited by ≥200 μM sulfide. These results indicate that sulfide stimulation of nO_2 may be correlated to environmental exposure to sulfide. Serotonin, a neurohormonal stimulant of ciliary beating, further increased sulfide-stimulated nO_2, possibly in support of energy demand. Sulfide-stimulated nO_2 was negligible in boiled gills and was 61% inhibited by cyanide, implicating the participation of mitochondrial electron flux. Mitochondrial cytochromes *c* and oxidase oxidation/reduction state changed little at <500 μM sulfide, but reduction occurred at 500–2000 μM sulfide, suggesting that although cytochrome oxidation/reduction state may be regulated in the face of increased electron flux, regulation may fail at inhibitory sulfide levels. Sulfide-stimulated nO_2 may represent a detoxification mechanism in *G. demissa*.

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Introduction

Hydrogen sulfide is common in marine reducing environments such as anoxic sediments, sewage sludge outfalls, deep-sea hydrothermal vents, and cold seeps. Because sulfide (the sum of H_2S, HS^-, and $S^=$ unless otherwise specified) is a potent inhibitor of mitochondrial cytochrome oxidase (Nicholls, 1975; Wilson and Erecinska, 1978; National Research Council, 1979) and can render hemoglobin nonfunctional through the formation of sulfhemoglobin (Berzofsky *et al.*, 1971; Carrico *et al.*, 1978), it is potentially toxic to a variety of aerobic organisms. In invertebrate/sulfur-oxidizing bacteria symbioses that inhabit reducing sediments and deep-sea hydrothermal vents, the host may exhibit mechanisms to reduce sulfide toxicity; these mechanisms include oxidation of sulfide to less toxic species and binding of sulfide to vascular and intracellular carrier proteins (reviewed in Somero *et al.*, 1989; Fisher, 1990; Childress and Fisher, 1992). Organisms without sulfur-oxidizing symbionts must also detoxify sulfide to survive in marine reducing environments. Catalysis of sulfide oxidation has been demonstrated in a variety of nonsymbiotic invertebrates and may involve mechanisms such as sulfide oxidases and nonenzyme catalysts such as metals and organic compounds (reviewed in Somero *et al.*, 1989).

An additional mechanism for sulfide detoxification may occur in mitochondria, which may harness the energy from sulfide oxidation. Sulfide oxidation coupled with ATP production has been demonstrated in mitochondria isolated from the symbiotic clam *Solemya reidi*, with thiosulfate as the major endproduct (Powell and Somero, 1986; O'Brien and Vetter, 1990), from the liver

of a sulfide-tolerant fish (Bagarinao and Vetter, 1990), and from the symbiont-free burrowing polychaete *Heteromastus filiformis* (Oeschger and Vismann, 1994). It is not known if mitochondrial sulfide oxidation occurs in intact tissues, if the energy potentially gained from mitochondrial sulfide oxidation contributes significantly to cellular work demands (Doeller, 1995), or how widespread such a pathway may be among organisms that encounter sulfide.

In the present study, we investigated the interaction between sulfide and mitochondria in the intact excised gill of the estaurine ribbed mussel *Geukensia demissa* (formerly *Modiolus demissus*). These mussels are commonly found partially or totally buried in sediments of *Spartina* grass tide marshes (Kuenzler, 1961). Animals at the sediment surface may exhibit air-gaping behavior by keeping their valves open during low tide (Lent, 1968). Porewater around sediment associated with *Spartina* can contain high (millimolar) concentrations of sulfide (Carlson and Forrest, 1982; King *et al.*, 1982; this study), thus *G. demissa* is most likely subject to chronic sulfide exposure. The objectives of the present study were to characterize environmental sulfide concentrations where *G. demissa* was collected; to measure oxygen consumption rates in excised gills as a function of ambient sulfide exposure in the presence and absence of the neurohormone serotonin, which stimulates ciliary beating and hence ATP demand (Clemmesen and Jørgensen, 1987); and to determine the effects of sulfide and serotonin on mitochondrial cytochrome oxidation/reduction state in excised gills. For comparison, parallel experiments were conducted with excised gills from *Mytilus galloprovincialis* or *Mytilus edulis* collected from habitats in which sulfide concentrations are most likely negligible.

Materials and Methods

Mussel collection and maintenance

Geukensia demissa (60–70 mm shell length) was collected during low to mid tide from *Spartina* grass beds on Dauphin Island, Alabama. In the laboratory, the mussels were maintained in recirculating seawater aquaria at 23°C. Some aquaria were partially filled with sediment obtained from the collection site and periodically seeded with dried *Spartina* pieces to stimulate sulfide production. The *G. demissa* specimens in these mudtanks were partially buried in the sediment to maintain their exposure to sulfide-enriched conditions. Animals were maintained in artificial seawater (Tropic Marin) at 24–30 ppt to correspond with collection salinity. *Mytilus galloprovincialis* (60 mm in shell length) obtained from offshore oil platforms in the Santa Barbara Channel, California, and *M. edulis* (50–60 mm in shell length) obtained from pilings of the Goleta Pier, California, were maintained for several weeks in recirculating seawater aquaria at 23°C and 30–35 ppt salinity prior to experiments. Experiments were performed at 20°C at maintenance salinity.

In situ *sulfide measurements*

The reaction of sulfide with 2,2′-dipyridyl disulfide (2-PDS) to form 2-thiopyridone, detected spectrophotometrically at 343 nm, is a simple and sensitive assay for determinating hydrogen sulfide in aqueous solution (Svenson, 1980). Sulfide standards treated with 2-PDS were found to have stable absorbances for at least 6 days when kept at room temperature or in the refrigerator. Sulfide was not lost from standards subjected to our collection protocol (described below). Hemolymph of freshly collected *G. demissa* and 1 m*M* sodium azide added to prevent bacterial growth did not interfere with the detection of internal sulfide standards.

Sediment porewater was sampled in May 1995 at *G. demissa* collection sites. A 50-cm piece of 18-gauge stainless steel tubing with small perforations near the tip and a female locking Luer connection at the base was attached to a 10-ml plastic syringe and inserted several centimeters into the sediment where many of the buried *G. demissa* were found. Aliquots (1–10 ml) of porewater were collected into the syringe, then filtered through a 13-mm glass-fiber filter mounted in a Millipore swinnex syringe filter holder. After the first few drops of filtered porewater were discarded, 50–200-μl aliquots were immediately pipetted into a 2.0-ml assay mixture containing 0.75 m*M* 2-PDS, 50 m*M* sodium phosphate, and 1 m*M* sodium azide (pH 6.5) for later determination of hydrogen sulfide. Internal fluid (a mixture of hemolymph and seawater contained in the mantle cavity) was obtained from specimens of *G. demissa* within minutes of their removal from the sediment by using a syringe with the needle inserted between the valves. Aliquots (250–500 μl) of this fluid were pipetted into the assay mixture for determination of sulfide. The sulfide in these samples was quantified the following day in the laboratory. Sodium sulfide standards prepared and added to the assay mixture in the field were used to construct a standard curve.

Excised gill closed-chamber respirometry

Sample preparation. Intact demibranchs were excised and placed in dishes containing filtered (0.45 μm) artificial seawater (FASW) for at least 1 h to allow mucus to clear from the gill surface. Gill pieces (0.1–0.2 g wet weight) were then placed on stainless steel screen holders (Doeller *et al.*, 1990) and inserted into a closed dual-chamber respirometer (Oroboros Oxygraph; Paar KG,

Graz, Austria) containing 6 ml stirred (500 rpm), air-equilibrated FASW at 20°C. After experiments, gill pieces were dried for at least 48 h at 70°C and weighed. Oxygen consumption rates are presented as mean pmol O_2 s^{-1} mg (dry weight)$^{-1}$ ± standard deviation.

Effects of sulfide on gill oxygen consumption rate. Three determinations of normoxic oxygen consumption rate were made, each over roughly 5-min intervals. Oxygen concentration in the respirometer was maintained close to air-saturation by periodically adding oxygen-equilibrated FASW through the injection port with a microliter syringe (Hamilton). Gills were then exposed to sulfide by addition of microliter aliquots of freshly made 30 m*M* sodium sulfide in FASW. Measurements were taken for at least 5 min at sulfide concentrations of 10–2000 μM. For each sulfide concentration, the rate of oxygen consumption due to spontaneous oxidation of sulfide was determined using respirometer chambers without excised gills. These background rates, which were ≤10% of the rates observed with freshly collected gills for sulfide concentrations of 500 μM or less, were subtracted from experimental oxygen consumption rates. Initial (stated) sulfide concentrations dropped less than 5% due to spontaneous sulfide oxidation during the course of a measurement. Oxygen consumption rates were measured within the pO_2 range of 156 torr to 19 torr and were found to be independent of pO_2 within this range (data not shown).

Treatments. The effects of sulfide on gill oxygen consumption rate were determined using groups of mussels as follows: *G. demissa* within 4 days from field collection (termed freshly collected), *G. demissa* maintained in aquaria with sulfide-enriched sediment for approximately 2 months (termed mudtank-maintained), *G. demissa* maintained in aquaria without sulfide sediment for about 2 months (termed sulfide-free), and *Mytilus galloprovincialis* or *M. edulis* with no history of sulfide exposure. This allowed an assessment of the effects of ambient sulfide exposure. To determine the relationship between energy demand and sulfide effects on oxygen consumption rate, the neurohormone serotonin (10 μM) was used to stimulate ciliary beating (Clemmesen and Jørgensen, 1987). The cytochrome oxidase inhibitor cyanide was used to determine mitochondrial participation in sulfide-mediated oxygen consumption. Sulfide-mediated oxygen consumption rate of gill pieces boiled for 10 min was measured to determine the proportion that is protein-based.

Excised gill optical spectrophotometry

Sample preparation. Excised intact demibranchs were kept in FASW for at least 1 h. Demibranch pieces, about 1 cm^2, were mounted between two frames, one made from stainless steel screen (60 mesh; outer dimension 15 × 15 mm; inner dimension 8 × 6 mm) and one from black plastic (outer dimension 14 × 12 mm; inner dimension 7 × 4 mm). Mounted gills were placed in a water-jacketed (20°C) cuvette containing 6 ml FASW buffered with 25 m*M* Tris (pH = 8.0) below a 6-ml gas space. A sulfide-insensitive polarographic oxygen sensor (Orbisphere model 2120, Geneva, Switzerland) as the floor of the cuvette was used to continuously measure solution pO_2. A stopper that sealed the top of the cuvette contained a stirring motor and stainless steel turbine (460 rpm), inflow and outflow gas lines, and an injection port. Optical spectra were acquired with a recording spectrophotometer equipped with a scattered transmission accessory and a digital data acquisition and analysis system (Cary model 14; Aviv Associates, Lakewood, NJ). Optical spectra were recorded from 400 to 650 nm at 1-nm intervals. Effects of treatments on oxidation/reduction state of mitochondrial cytochromes were determined from difference spectra of gills under treatment conditions minus the same gills under air-saturated seawater conditions. Spectral features associated with cytochrome reduction observed in these difference spectra were quantified by determining optical density differences (ΔODs) between absorption maxima and a baseline obtained by visually fitting data points adjacent to peaks to a straight line. Absorption maxima observed at 550 nm were ascribed to cytochrome *c*. Absorption maxima observed at 605 nm in *G. demissa* gills and at 610 nm in *M. edulis* gills were ascribed to cytochrome oxidase. Percent reduction of cytochromes was calculated by comparing treatment ΔOD to ΔOD taken from difference spectra of gills exposed to conditions under which mitochondrial cytochromes are maximally reduced (addition of 1 mg ml^{-1} sodium dithionite to the medium) minus gills under air-saturated conditions.

Effects of sulfide on mitochondrial cytochrome oxidation/reduction state. Optical spectra of air-equilibrated gills were acquired while passing humidified air through the spectrophotometer cuvette at a flow rate of 100 ml min^{-1}, regulated by a mass flow controller (Tylan, Carson, CA). Gills were then exposed to increasing concentrations of sulfide (20–2000 μM) by adding microliter aliquots of freshly made 30 m*M* sodium sulfide solution through the injection port of the cuvette with a Hamilton syringe. Based on a pK of 6.59 for the dissociation of sulfide in 35 ppt seawater at 20°C (Millero *et al.*, 1988) and a solubility of 146 μM H_2S torr^{-1} (Millero, 1986), a sulfide concentration of 20–2000 μM at pH 8.0 corresponds to a pH_2S of 0.005–0.51 torr. To prevent loss of H_2S from the medium, hydrogen sulfide gas was metered into the humidified air by using a syringe pump (Harvard) to match pH_2S with sulfide concentration. Oxygen partial pressure was found to stabilize within 10 to 15

min after exposure to each sulfide concentration, at which time optical spectra were acquired.

Treatments. Groups of mussels similar to those used in closed-chamber respirometric experiments were used in spectrophotometric experiments: freshly collected *G. demissa*, sulfide-free *G. demissa*, and *M. edulis* collected from a sulfide-free environment and maintained without sulfide. The neurohormone serotonin (10 μM) was used to determine the relationship between energy demand and cytochrome oxidation/reduction state with and without sulfide.

Results

In situ *hydrogen sulfide measurements*

Sediment porewater was collected at several intertidal sites within the *Spartina* marsh where *Geukensia demissa* seemed abundant. All sites were among *S. alterniflora*. At sampling time during midtide, sediments were either exposed to air or covered by 20–40 cm of overlying seawater. Sulfide concentration in porewater samples ranged from 1.3 mM to 8.1 mM, with a mean value of 4.8 mM ($n = 6$). Fluid consisting of a mixture of hemolymph and seawater contained within the mantle cavity of 10 mussels (≈60–70 cm shell length) collected from a clump of *S. alterniflora* roots had neither a sulfide odor nor a detectable sulfide concentration. The sulfide concentration in the porewater sample collected nearest to these mussels was 1.3 mM. The absence of detectable sulfide in the mantle cavity fluid may have resulted from tissue sulfide oxidation or from exchange of mantle cavity fluid with overlying seawater possibly brought on by the mechanics of dislodging the animal during collection.

Sulfide stimulation of oxygen consumption rate in gills of freshly collected Geukensia demissa

In the absence of sulfide, *G. demissa* gills exhibited a mean normoxic oxygen consumption rate (nO_2) of 15.0 ± 2.8 pmol O_2 s^{-1} mg^{-1} ($n = 6$; Fig. 1A). The addition of sulfide stimulated nO_2 to a maximum near 50 pmol O_2 s^{-1} mg^{-1} ($n = 6$) at 200–500 μM sulfide (Fig. 1A). At 1000 μM sulfide, nO_2 averaged 37.3 ± 7.6 ($n = 6$; Fig. 1A).

Addition of 10 μM serotonin in the absence of sulfide resulted in a 1.8-fold stimulation of nO_2 to 27.5 ± 5.6 ($n = 5$; Fig. 1A). As before, sulfide stimulated nO_2, which reached a maximum of 78.1 ± 12.0 pmol O_2 s^{-1} mg^{-1} ($n = 5$) at 200 μM sulfide (Fig. 1A). At 1000 μM sulfide, nO_2 remained elevated at 55.9 ± 10.8 ($n = 5$; Fig. 1A).

Sulfide-stimulated oxygen consumption rate in gills of mudtank-maintained Geukensia demissa

Gills of mudtank-maintained *G. demissa* exhibited a mean nO_2 of 10.8 ± 2.5 pmol O_2 s^{-1} mg^{-1} ($n = 10$; Fig. 1A). At 200 μM sulfide, sulfide-stimulated nO_2 was 55.3 ± 9.4 pmol O_2 s^{-1} mg^{-1} ($n = 5$), comparable to the rate exhibited by gills of freshly collected *G. demissa* at 200 μM sulfide (Fig. 1A). Thus the sulfide-stimulated nO_2 at 200 μM sulfide does not decline over time, unlike the rate observed in animals kept in the absence of sulfide for a similar time period (see below). This result indicates that animals kept in aquaria with sulfide-enriched sediment maintain a capacity for sulfide oxidation similar to that of freshly collected animals.

Effects of boiling on sulfide-stimulated oxygen consumption rate by gills of mudtank-maintained Geukensia demissa

Geukensia demissa gills boiled for 10 min exhibited negligible rates of sulfide-stimulated oxygen consumption over the entire range of sulfide concentrations (50–1000 μM; Fig. 1A).

Sulfide-stimulated oxygen consumption rate in gills of Geukensia demissa *maintained without sulfide for about 2 months*

Strikingly different responses to sulfide were observed between gills of freshly collected mussels and gills of mussels maintained in aquaria without sulfide sediment. Mean nO_2 of gills from sulfide-free *G. demissa* in the absence of sulfide was 12.7 ± 3.2 pmol O_2 s^{-1} mg^{-1} ($n = 12$; Fig. 1B), similar to that of gills from freshly collected mussels. Maximum sulfide-stimulated nO_2 exhibited at 200 μM sulfide, was 30.0 ± 4.9 pmol O_2 s^{-1} mg^{-1} ($n = 6$; Fig. 1B), 60% of that exhibited by gills of freshly collected mussels. At greater than 200 μM sulfide, nO_2 noticeably declined, and at 1000 μM sulfide they were no different than control ($P = 0.27$; ANOVA; Fig. 1B).

Treatment with 10 μM serotonin resulted in a 2.0-fold stimulation of nO_2 to 27.5 ± 5.6 pmol O_2 s^{-1} mg^{-1} ($n = 7$; Fig. 1B). Maximum sulfide-stimulated nO_2, observed at 100 μM sulfide, was 45.1 ± 10.1 pmol O_2 s^{-1} mg^{-1} ($n = 7$; Fig. 1B), 59% of that exhibited by gills of freshly collected mussels at 200 mM sulfide. At greater than 100 μM sulfide, nO_2 declined dramatically, and at 1000 μM sulfide they were 13.2 ± 5.7 pmol O_2 s^{-1} mg^{-1} ($n = 7$; Fig. 1B), 48% of the serotonin-stimulated control value ($P < 0.05$; ANOVA).

Sulfide-stimulated oxygen consumption rate in gills of Mytilus galloprovincialis *collected from a sulfide-free habitat*

The mean nO_2 exhibited by *M. galloprovincialis* gills in the absence of sulfide was 15.0 ± 2.3 pmol O_2 s^{-1} mg^{-1} ($n = 5$; Fig. 1C), similar to rates exhibited by *G. demissa* gills under the same conditions (Fig. 1A, B). Exposure

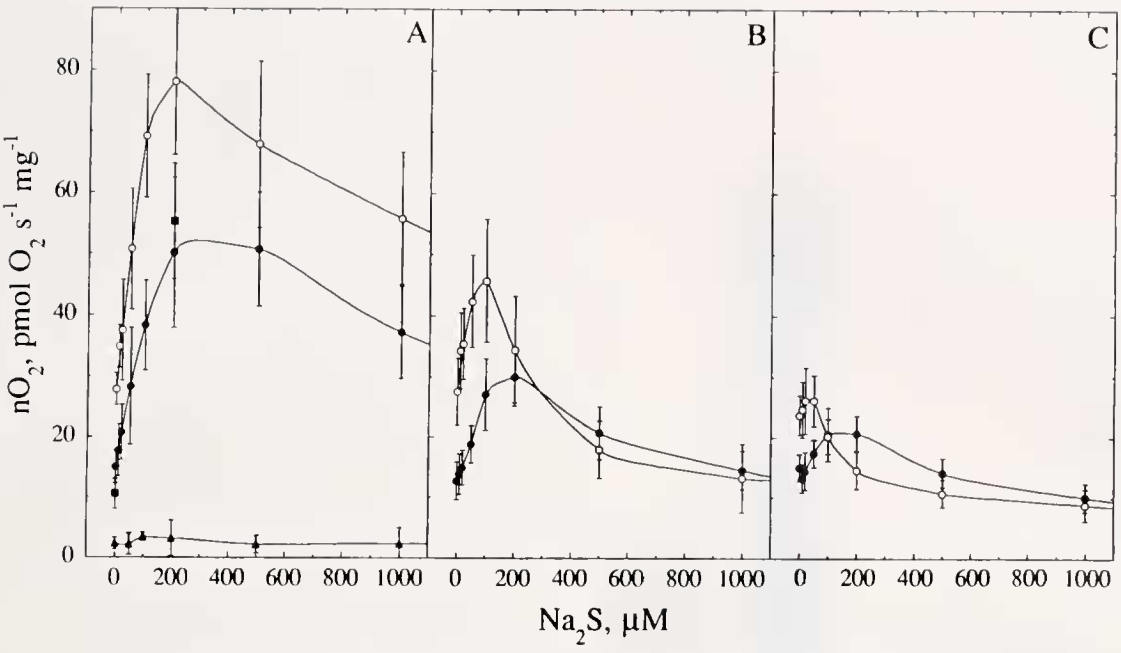

Figure 1. Oxygen consumption rate (nO_2) of excised mussel gills as a function of sulfide concentration, in the presence (open symbols) and absence (closed symbols) of serotonin. Rates are given as mean ± SD. (A) Gills of freshly collected *Geukensia demissa* (circles); gills of mudtank-maintained *G. demissa* (squares); boiled *G. demissa* gills (diamonds). (B) Gills of sulfide-free *G. demissa* (C) Gills of *Mytilus galloprovincialis*.

to sulfide slightly but significantly increased nO_2, with a maximum near 20 pmol O_2 s^{-1} mg^{-1} (n = 5) observed at 100–200 μM sulfide ($P < 0.05$ for both treatments; ANOVA; Fig. 1C). At 1000 μM sulfide, nO_2 was 10.1 ± 2.4 pmol O_2 s^{-1} mg^{-1} (n = 5; Fig. 1C), 67% of the control value.

M. galloprovincialis gills exposed to 10 μM serotonin in the absence of sulfide exhibited a 1.6-fold stimulation of nO_2 to 23.7 ± 3.3 pmol O_2 s^{-1} mg^{-1} (n = 5; Fig. 1C). Oxygen consumption rates were significantly inhibited by ≥100 μM sulfide ($P < 0.05$ for 100 μM and 200 μM sulfide; ANOVA), and at 1000 μM sulfide were 8.9 ± 2.6 pmol O_2 s^{-1} mg^{-1} (n = 5; Fig. 1C), 38% of the serotonin-stimulated control value.

Effect of cyanide on sulfide-stimulated oxygen consumption rate by gills of mudtank-maintained Geukensia demissa

To test for mitochondrial participation in sulfide-stimulated nO_2, the effect of cyanide, an inhibitor of mitochondrial cytochrome oxidase, was determined in gills of mudtank-maintained *G. demissa* (Fig. 2). Control nO_2 of these gills (see Fig. 1A) was inhibited by more than 90% in the presence of 1 mM potassium cyanide (Fig. 2). Oxygen consumption rate in the presence of 200 mM sulfide (see Fig. 1A) was inhibited by cyanide to 21.6 ± 7.2 pmol O_2 s^{-1} mg^{-1} (n = 5; Fig. 2), representing a 61% inhibition of the rate in the absence of cyanide.

Effects of sulfide on the oxidation/reduction state of cytochromes c *and oxidase in mussel gills*

Difference spectra of *G. demissa* gills exposed to sodium dithionite (1 mg ml^{-1}) minus the same gills equilibrated with air showed reduced minus oxidized mitochondrial cytochromes *b* and *c* and oxidase (Fig. 3, upper line). Under normoxic conditions, addition of sulfide at 20–200 μM did not alter gill mitochondrial cytochrome oxidation/reduction state, as indicated by difference spectra of gills in the presence of 50 μM sulfide minus the same gills in the absence of sulfide (Fig. 3, lower line). Steady-state nO_2 of gills in the spectrophotometer cuvette (data not shown) increased in response to sulfide in a manner similar to that of gills in the closed-chamber respirometer (Fig. 1); however, high sulfide concentrations (1000 μM) were required to cause cytochrome reduction (Fig. 3, middle line).

Reduction of cytochromes *c* and oxidase in *G. demissa* gills neared 50% at sulfide concentrations of 500 μM or greater (Figs. 4A, B; 5A, B), and neither cytochrome reached full reduction at sulfide concentrations up to 2000 μM. Results from gills of freshly collected and sulfide-free *G. demissa* were similar, although cytochrome

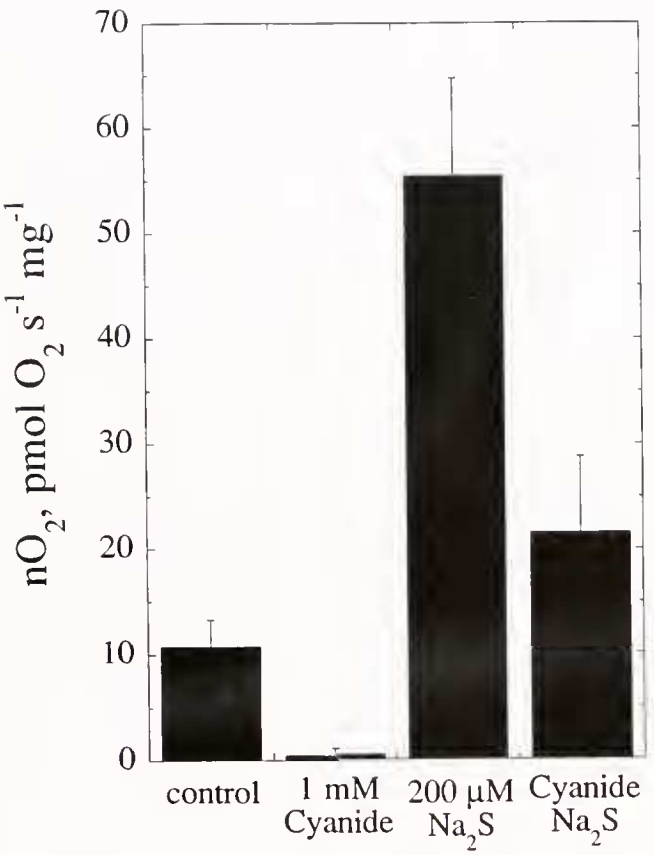

Figure 2. Oxygen consumption rate (nO_2) of excised gills of mudtank-maintained *Geukensia demissa* in the presence of sulfide (200 μM Na_2S) and cyanide (1 mM). Rates are given as mean ± SD.

c in gills of freshly collected *G. demissa* exhibited decreased reduction in the presence of serotonin (Fig. 4A), and the percentage reduction of cytochrome oxidase was slightly higher in gills of sulfide-free mussels at sulfide concentrations of 500 μM or greater (Fig. 5B). Little reduction of cytochromes *c* and oxidase of *M. edulis* gills was observed below 500 μM sulfide, and less than 100% reduction was observed at 500–2000 μM sulfide (Figs. 4C, 5C); these results were similar to those obtained with *G. demissa* gills.

Addition of 10 μM serotonin to *G. demissa* and *M. edulis* gills increased the steady-state rates of oxygen consumption (data not shown) in a manner similar to that exhibited by gills in the closed-chamber respirometer (Fig. 1) but produced little detectable change in cytochrome redox state (Figs. 4, 5). Changes in percentage reduction of cytochromes *c* and oxidase in response to sulfide were similar to results observed in the absence of serotonin (Figs. 4, 5).

Discussion

Animals that routinely encounter sulfide in their habitats possess mechanisms to oxidize sulfide to less toxic forms, thus preventing sulfide poisoning of aerobic respiration (Somero *et al.*, 1989). We investigated two genera of mussels exposed to different environmental levels of sulfide to determine whether a correlation exists between environmental sulfide exposure, oxygen consumption rate, and cytochrome redox state. Ciliated gills, which exist at the interface between animal and environment, were used as models of aerobically active tissues that are sensitive to environmental conditions.

The oxygen consumption rates of excised gills of *Geukensia demissa, Mytilus galloprovincialis,* and *M. edulis* measured before and after stimulation with 10 μM serotonin were similar to those previously reported for gills of *M. edulis* under similar conditions (Clemmesen and Jørgensen, 1987; Doeller *et al.*, 1993). However, sulfide effects on oxygen consumption rate of gill tissue appeared to be correlated with environmental sulfide exposure. Sulfide-stimulation of oxygen consumption rate was greatest in gills of *G. demissa* with a history of sulfide exposure—that is, in freshly collected mussels and mussels maintained in sulfide-enriched mudtanks. Gills of *G. demissa* exposed to sulfide-free conditions for more than 2 months exhibited substantially reduced rates of sulfide-stimulated oxygen consumption. Gills of *M. galloprovincialis,* a species that does not routinely encounter high sulfide levels, exhibited the lowest rates of sulfide-stimulated oxygen consumption. The reduced level of sulfide-stimulated oxygen consumption in animals not exposed to sulfide may reflect the inactivation or lack of a metabolic pathway involved in sulfide oxidation. The presence of a small amount of sulfide-stimulated oxygen consumption in *M. galloprovincialis* may be the result of a general tissue capacity for sulfide oxidation, as discussed by Anderson (1995) in reference to sulfide oxidation capacity in *M. californianus* gills, and may not reflect adaptation to environmental sulfide exposure.

Sulfide-stimulation of oxygen consumption rate may reflect oxidation of sulfide to less toxic forms such as thiosulfate, sulfite, sulfate, or elemental sulfur, and this process may function in part to protect aerobic metabolism from sulfide poisoning of cytochrome oxidase. Mitochondria isolated in the uncoupled state from *G. demissa* gills showed inhibition of oxygen consumption beginning at 5 μM sulfide (Lee, unpub.), suggesting that if cytochrome oxidase is as sensitive in the intact tissue as it is in isolated uncoupled mitochondria, intracellular sulfide concentration must be maintained at a low level. The oxygen consumption results reported here are consistent with a protective function of sulfide oxidation.

The enhanced capacity for sulfide oxidation exhibited by *G. demissa* with a history of sulfide exposure may be due to non-enzyme–catalyzed sulfide oxidation, sulfide-oxidase enzymes, or mitochondrial oxidation of sulfide. Because boiled gills exhibited no sulfide-stimulated oxy-

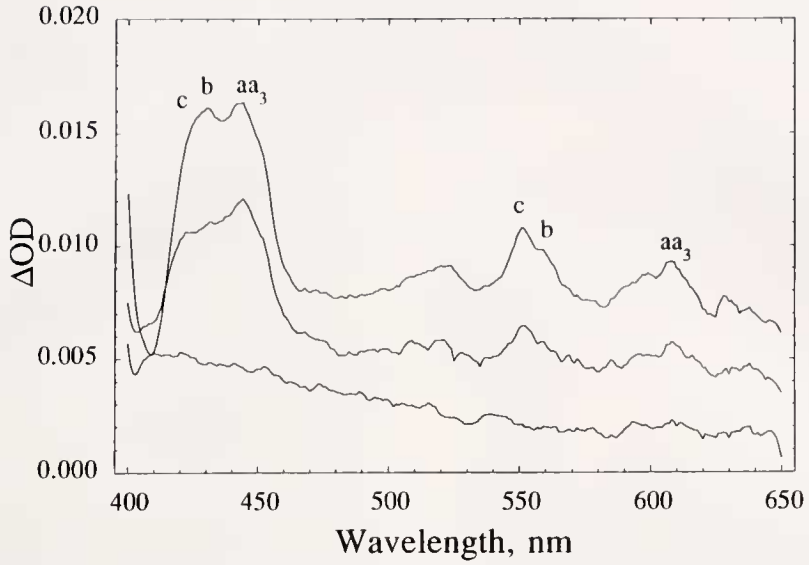

Figure 3. Difference spectra of gills of freshly collected *Geukensia demissa:* in seawater with 1 mg ml^{-1} dithionite minus the same gills in air-equilibrated seawater (top spectrum); in seawater with 1000 μM Na_2S minus the same gills in air-equilibrated seawater (middle spectrum); and in seawater with 50 μM Na_2S minus the same gills in air-equilibrated seawater (bottom spectrum). The top difference spectrum shows the full complement of reduced minus oxidized mitochondrial cytochromes oxidase (aa_3), *b*, and *c*.

gen consumption, mechanisms responsible for sulfide oxidation may involve sulfide-oxidase enzymes or mitochondria since both are probably more sensitive to heat denaturation than are sulfide-oxidizing catalysts such as metal ions or organic compounds (Chen and Morris, 1972).

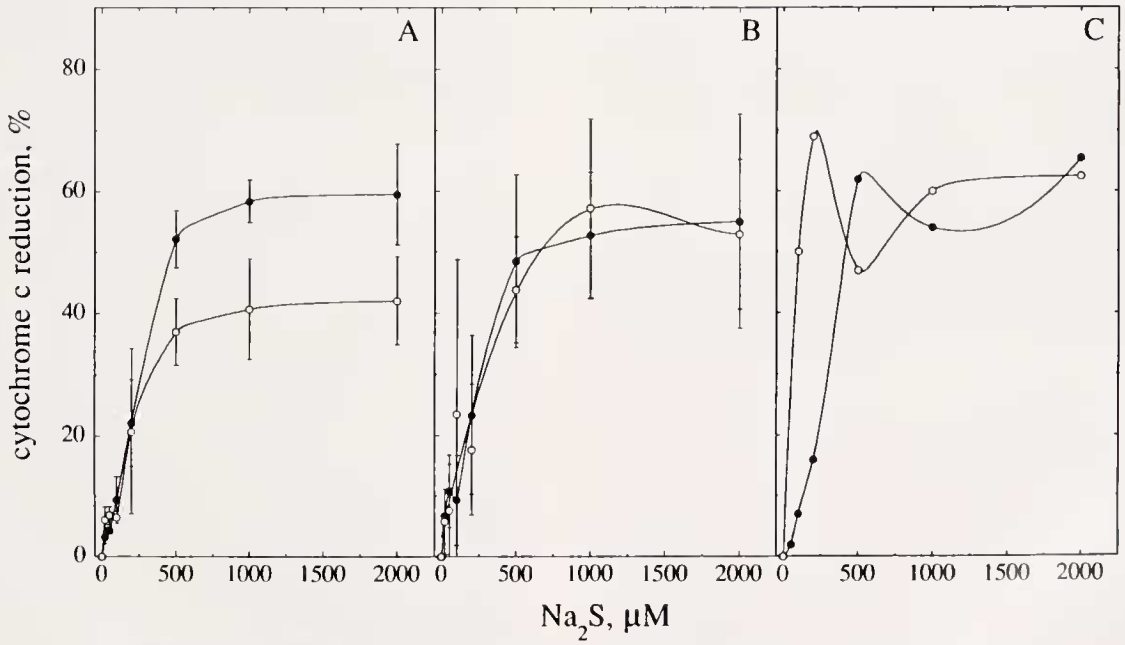

Figure 4. Percentage reduction of cytochrome *c* in mussel gills in the presence (open circles) and absence (closed circles) of serotonin as a function of sulfide concentration. (A) Gills of freshly collected *Geukensia demissa.* (B) Gills of sulfide-free *G. demissa.* (C) Gills of *Mytilus edulis.* Values are given as mean ± SD (n = 3) in A and B, and as the average of two experiments in C.

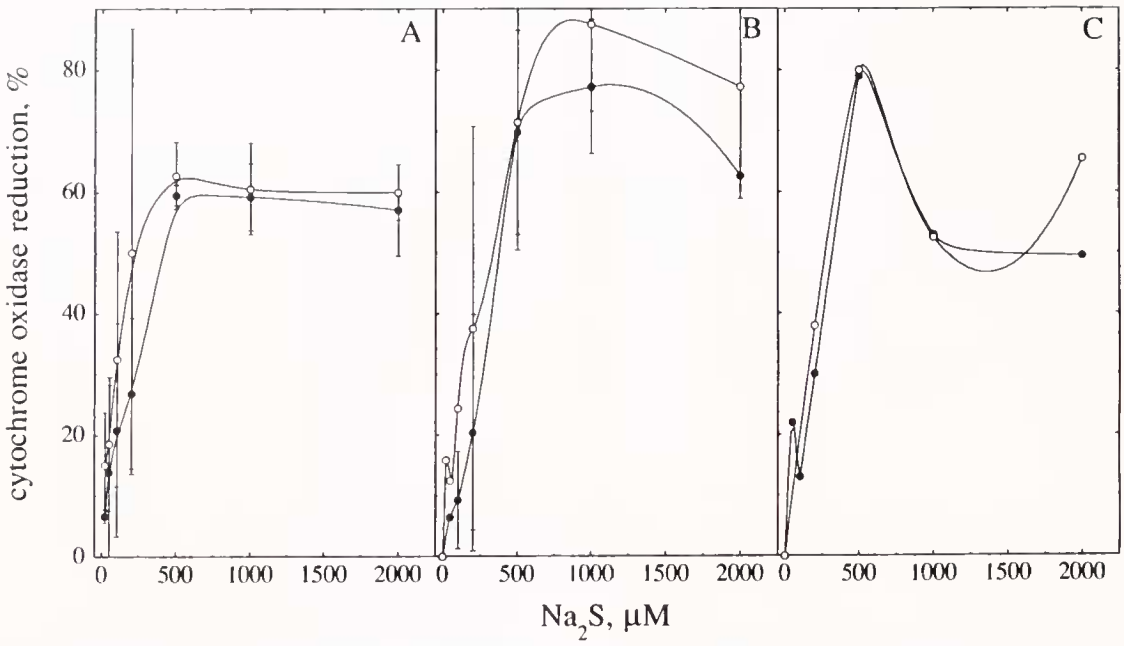

Figure 5. Percentage reduction of cytochrome oxidase (aa_3) in mussel gills in the presence (open circles) and absence (closed circles) of serotonin as a function of sulfide concentration. (A) Gills of freshly collected *Geukensia demissa* (B) Gills of sulfide-free *G. demussa* (C) Gills of *Mytilus edulis*. Values are given as mean ± SD (n = 3) in A and B, and as the average of two experiments in C.

Results from experiments with cyanide and serotonin indicate that part of the sulfide-stimulated oxygen consumption exhibited by *G. demissa* gills may be mitochondrial in nature. Cyanide, an inhibitor of cytochrome oxidase, reduced the rate of sulfide-stimulated oxygen consumption by 61%, indicating that treatment to inhibit mitochondrial electron flux reduces the rate of sulfide oxidation. In buffered distilled water, pH 9 and 25°C, cyanide at 20 m*M* inhibits spontaneous oxidation of 20 m*M* sulfide by greater than 90% (Chen and Morris, 1972). Under our experimental conditions of 1 m*M* cyanide, pH 8, and 20°C, oxidation of 200 μ*M* sulfide in chambers containing filtered seawater without gill tissue (called background oxygen consumption) was inhibited by only about 40%. Therefore, the effect of cyanide on spontaneous sulfide oxidation in our system is small. Since cyanide inhibited more than 90% of gill oxygen consumption in the absence of sulfide, this respiration appears to occur mostly *via* mitochondria cytochrome oxidase. However, mechanisms responsible for the observed cyanide-insensitive respiration in the presence of sulfide may include a salicylhydroxamic acid (SHAM)-sensitive pathway in which electrons are transferred to oxygen *via* an alternative oxidase, as has been shown to occur in sulfide-exposed mitochondria isolated from the lugworm *Arenicola marina* (Völkel and Greishaber, 1996). Preliminary experiments with *G. demissa* gills show that serotonin-stimulated respiration that is partially inhibited by 1 m*M* cyanide is then fully inhibited by the addition of 50 μ*M* SHAM (Kraus and Doeller, unpub.).

The nearly 2-fold increase in oxygen consumption rate exhibited by mussel gills in the presence of serotonin most likely results from the increased energy demand associated with stimulated ciliary beating. If sulfide oxidation is linked to oxidative phosphorylation, with electrons from sulfide being used for ATP production, then the rates of sulfide oxidation should be enhanced when energy demand is higher, as in the presence of serotonin. Our data confirm this prediction. The sulfide-stimulated increase in oxygen consumption rate in gills of freshly collected *G. demissa* was 45% higher in the presence of serotonin than in its absence, indicating that sulfide may participate in ATP production. The proportion of ATP produced from sulfide oxidation is currently being addressed (Gaschen, Kraus, and Doeller, unpub.). Sulfide delivery to gill cells may also be enhanced by the serotonin-stimulated increase in ciliary beating as a result of reduced boundary layers, as discussed by Wright (1979) and Doeller *et al.* (1993) for the enhanced delivery of amino acids and oxygen, respectively, to mussel gills in the presence of serotonin.

In contrast to freshly collected *G. demissa* gills, gills from *G. demissa* maintained without sulfide or gills from *M. galloprovincialis* did not exhibit sulfide stimulation of oxygen consumption rate after treatment with seroto-

nin. In fact, these serotonin-treated gills exhibited inhibition of oxygen consumption at sulfide concentrations that caused slight stimulation of non-serotonin–treated oxygen consumption. Serotonin-mediated enhancement of electron flux through cytochrome oxidase in these gills may make components involved in oxygen-consuming pathways more sensitive to sulfide inhibition; this is analogous to the change in sensitivity to oxygen exhibited by mitochondria under different metabolic states (Wilson *et al.*, 1988).

Changes in the redox state of mitochondrial cytochrome in mussel gills indicate that experimental conditions have caused differential electron flux through the cytochromes. Our data show little change in cytochrome redox state in gills of either *G. demissa* or *M. edulis* at 0–200 μM sulfide, concentrations that greatly stimulate oxygen consumption rate in gills of freshly collected *G. demissa*. Cytochromes were reduced by 50% or higher only at $\geq$ 500 μM sulfide, concentrations at which oxygen consumption rate showed inhibition. At these high sulfide concentrations, serotonin-stimulated gills of freshly collected *G. demissa* exhibit comparatively high rates of oxygen consumption—indicating high electron flux through the electron transport chain—and this may have contributed to the observed redox state of cytochrome *c*. Wilson *et al.* (1988) showed that cytochrome *c* in mitochondria isolated from rat liver exhibits oxygen-dependent redox changes that may influence the rate of oxidative phosphorylation.

Little change in redox state suggests either that the rates of supply and exit of electrons to and from the cytochromes were in balance or that electron flux did not change. If the rates were in balance, then the large increase in the rate of oxygen consumption by *G. demissa* gill may indicate that sulfide oxidation is in part mitochondrial and largely regulated. Oxygen consumption rates in the presence of cyanide and serotonin support the scenario that sulfide oxidation is coupled to ATP production. The possibility also exists that sulfide-stimulated mitochondrial electron flux may be regulated but more or less uncoupled from phosphorylation (O'Brien and Vetter, 1990). On the other hand, if electron flux did not change, then the large increase in the rate of oxygen consumption by *G. demissa* gill must occur *via* pathways not coupled with ATP production.

In summary, the oxygen consumption rate of mussel gills is stimulated by sulfide to an extent that appears to be correlated with environmental exposure to sulfide. Sulfide-stimulated oxygen consumption may be the result of a metabolic pathway, perhaps associated with mitochondria, whose activation may be mediated by environmental sulfide. Whether this process is coupled with ATP production remains unknown, but sulfide-stimulated oxygen consumption by organisms inhabiting high-sulfide environments most likely represents a mechanism for sulfide detoxification.

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